Antiphospholipid syndrome: Determination of antibodies to beta 2 glycoprotein I (anti-β2GPI) by ELISA

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ANTIPHOSPHOLIPID ANTIBODIES are a heterogeneous group of immunoglobulins (autoantibodies) with specificity toward negatively charged phospholipids, including cardiolipin (diphosphatidylglycerol), phosphatidylserine, and phospholipid–protein complexes. The association of elevated serum levels of "autoimmune" antiphospholipid antibodies with recurrent venous or arterial thrombotic events, thrombocytopenia, and fetal loss (antiphospholipid syndrome) has been well established and is now widely recognized.[1]

These antiphospholipid antibodies frequently require the presence of a serum protein as cofactor for optimal in vitro binding activity. Cofactors show a high binding affinity for phospholipids forming phospholipid–protein complexes. The most studied cofactor is beta 2 glycoprotein I (β2GPI), a natural anticoagulant protein found in normal serum.[2] Experimental evidence suggests that "autoimmune" antiphospholipid antibodies may, in fact, recognize a newly formed antigenic site located at the cofactor molecule (i.e., β2GPI) when bound to phospholipids.[3] Continuing research on the role of protein cofactors has increased our understanding of the heterogeneous nature of antiphospholipid antibodies and their possible in vivo pathogenic role in thrombosis.[4]

It has been shown that the attachment of purified β2GPI to certain plastic surfaces of microplates (in the absence of phospholipids) can be used to detect antibodies to this cofactor. The binding of β2GPI to the plastic surface would produce antigenic sites similar to those produced when bound to phospholipids.[3] Anti-β2GPI antibodies detected by these systems are thought to be more specific for thrombosis than antibodies detected by classic antiphospholipid enzyme-linked immunosorbent assays or ELISAs (phospholipids bound to plastic, with exogenous source of cofactor, i.e., anticardiolipin and anti-phosphatidylserine assays).[5,6] Testing for anti-β2GPI antibodies in the clinical laboratory by ELISA is becoming increasingly valuable and provides additional clinically relevant results to assess patients for the antiphospholipid syndrome and/or the risk of thrombosis.

An ELISA test kit for the semiquantitative determination of antibodies specific for human β2GPI in serum has been developed. The technical and clinical performance of the assay are discussed here.

Table 1

Summary of anti-β2GPI assay precision

Serum sample	Unit range	Intraassay precision IgG (%CV)	Intraassay precision IgM (%CV)	Intraassay precision IgA (%CV)	Interassay precision IgG (%CV)	Interassay precision IgM (%CV)	Interassay precision IgA (%CV)
High +	>90	3.4	2.4	3.5	1.7	3.4	3.7
Mod. +	60–70	4.7	3.5	4.4	3.5	4.5	3.7
Low +	<50	4.3	3.8	4.7	4.0	6.1	4.7

Materials and methods

The anti-β2GPI test kit (**Corgenix, Inc.,** Westminster, CO) is performed as an indirect ELISA for the detection of IgG, IgM, or IgA antibody isotypes in human serum. One hundred microliters of diluted (1:50) patient samples, calibrator, and control sera are incubated in 96-well microplates coated with purified human β2GPI (purity >95% sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE). β2GPI is stabilized and noncoated areas are blocked to prevent nonspecific binding. Incubation at room temperature for 15 min allows anti-β2GPI antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum proteins by washing with phosphate-buffered saline (PBS)-Tween-20 solution, antibodies specific for human IgG, IgM, or IgA, labeled with horseradish peroxidase (HRP), are added to form complexes with the human antibodies bound to β2GPI. Following another washing step, a single solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) is added as a chromogenic substrate. The bound enzyme-antibody conjugate develops a colored signal proportional to the serum concentration (activity) of anti-β2GPI antibodies in the sample. Color development is stopped with the addition of 0.36 N sulfuric acid solution.

Results are obtained by reading the OD (optical density or absorbance) at 450 nm of each well in a spectrophotometer. Calibrator and control sera are provided, with the anti-β2GPI antibody concentrations expressed in units for IgG, IgM, and IgA. The OD values of the samples are multiplied by the conversion factor to obtain anti-β2GPI antibody concentrations in units. These units are traceable to available reference preparations.

The kit contains sufficient materials and reagents for 96 determinations including a calibrator and positive and negative control for IgG, IgM, or IgA anti-β2GPI antibodies. The assay features microplates with breakaway wells and color-coded liquid reagents for user convenience. Total incubation time is 40 min at room temperature (15, 15, 10 min).

Technical performance

Normal range

One hundred twenty serum samples from healthy blood bank donors were tested for anti-β2GPI antibodies to confirm the cutoff of the assay, which was established at 20 units for all three antibody isotypes. The specificity of the assay was 100% for IgG, 93% for IgM, and 96% for IgA. The assay measures antibody activity up to 200 units. Samples with anti-β2GPI values greater than 200 units should be further diluted and retested for more accurate measurement.

Precision

Three selected serum samples with known anti-β2GPI unit values (one low, one moderate, and one high) were assayed in 23 replicates on three different occasions. The mean intraassay and interassay coefficient of variation (%CVs) of the assay are shown in *Table 1*.

Clinical performance

When serum samples from 120 healthy blood donors were assayed for the presence of anti-β2GPI antibodies, a mean value of 2.1 ± 1.1 G units was obtained for IgG, 7.7 ± 9.5 M units for IgM, and 6.9 ± 5.7 A units for IgA antibodies (mean ± 1 SD).

Sensitivity for SLE

Serum samples from 40 consecutive (unselected) patients with systemic lupus erythematosus (SLE) were tested for anti-β2GPI antibodies. Nine of the samples (sensitivity of 22.5%) were positive for IgG with a mean value of 24.5 ± 49.6 G units, 8 samples (sensitivity of 20%) were positive for IgM with a mean value of 13.9 ± 18.1 M units, and 10 samples (sensitivity of 25%) were positive for IgA with a mean value of 42.6 ± 72.5 A units. Anti-β2GPI antibody levels of this SLE population were statistically different when compared to the healthy controls (single factor analysis of variance or ANOVA, $p < 0.001$ for all three isotypes). The value distribution for IgG anti-β2GPI antibodies is summarized in *Figure 1*.

A good positive correlation was found between IgG anti-β2GPI with both IgG anti-phosphatidylserine ($r = 0.928$) and IgG anti-cardiolipin ($r = 0.864$) antibody levels in this group of patients. IgM and IgA

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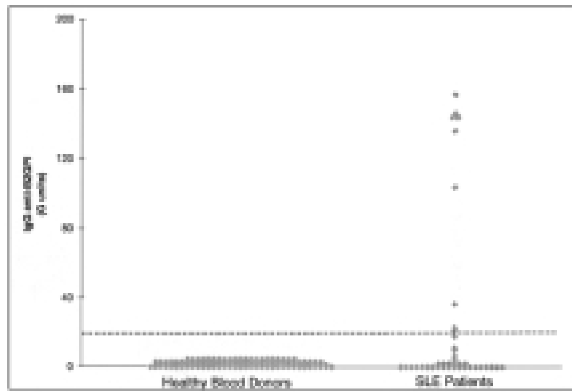

Figure 1 *Distribution of IgG anti-β2GPI antibodies in healthy controls and SLE patients measured by ELISA (broken line = normal cutoff).*

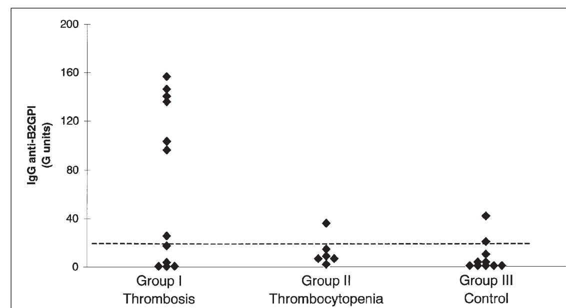

Figure 2 *Distribution of IgG anti-β2GPI antibodies in selected SLE patients with history as follows: group I—thrombosis, group II—thrombocytopenia, and group III—control, measured by ELISA (broken line = normal cutoff).*

anti-β2GPI antibodies showed a lower degree of correlation than IgG (r = from 0.4 to 0.5).

Sensitivity for thrombosis in SLE

Serum samples from 12 selected female patients with SLE who had a clinical history of thrombosis were evaluated for anti-β2GPI antibodies. Seven of the samples (sensitivity of 58%) were positive for IgG with a mean value of 69 ± 66.2 G units, five samples (sensitivity of 42%) were positive for IgM with a mean value of 24.2 ± 25.8 M units, and 8 samples (sensitivity of 67%) were positive for IgA with a mean value of 105.6 ± 97.5 A units for this group of selected SLE patients.

Many of these SLE patients also had a history of thrombocytopenia, and when their obstetric history was reviewed, there were 24 fetal losses out of 46 pregnancies recorded (55% abortion rate). The clinical history, along with anti-β2GPI results, indicates that these patients showed the clinical manifestation of the antiphospholipid syndrome.

Serum samples from six selected female patients with SLE who had a history of thrombocytopenia (no thrombosis) were tested for anti-β2GPI antibodies. Only one sample (17%) tested weak positive for IgG with a mean value of 12.7 ± 12.2 G units; none tested positive for IgM with a mean value of 6 ± 6.5 M units, and none tested positive for IgA with a mean value of 11.1 ± 5 A units for this group. No abortions out of 11 pregnancies were recorded in this group.

Serum samples from 10 selected female patients with SLE who were known not to have had thrombotic episodes, nor any other feature of this antiphospholipid syndrome (disease control), were tested for anti-β2GPI antibodies. Two of the samples (20%) were positive for IgG with a mean value of 8.5 ± 13.5 G units, one sample (11%) was positive for IgM with a mean value of 9.2 ± 12.1 M units, and one sample (11%) was positive for IgA with a mean value of 22.2 ± 49.4 A units. One sample in this group was moderate to strong positive for all immunoglobulin isotypes and was from the only patient who had an abortion of 18 pregnancies recorded.

Mean anti-β2GPI antibody levels from SLE patients with history of thrombosis were significantly higher than the disease control groups: single factor ANOVA, $p < 0.002$ for IgG, $p = 0.038$ for IgM, and $p = 0.005$ for IgA. The value distribution for IgG anti-β2GPI antibodies is summarized in *Figure 2*.

Discussion

The authors' results support previous reports that purified human β2GPI bound to microplates forms an epitope similar to that produced when β2GPI binds to phospholipids. Since most of the clinically relevant antiphospholipid antibodies have specificity toward the newly formed epitope, the laboratory determination and measurement of anti-β2GPI antibodies provided more specific information for thrombosis (antiphospholipid syndrome) in SLE patients.

Compared to the healthy controls, results show significantly higher anti-β2GPI antibody levels in SLE patients where elevated levels of antiphospholipid antibodies are most likely to be found. As previously described for anti-cardiolipin and anti-phosphatidylserine antibodies,[1] some healthy individuals may present elevated serum levels of anti-β2GPI antibodies (mostly IgM and IgA); however, the exact clinical significance of these antibodies is not yet well understood. They may be relevant as risk factors for the development of thrombosis (antiphospholipid syndrome). The clinical sensitivity of anti-β2GPI antibodies in the study's unselected SLE population (20–25%) is in agreement with previous studies and with the expected prevalence of other antiphospholipid antibodies in SLE patients.[1,2,6] Results with selected SLE patients show a strong association between elevated serum levels of anti-β2GPI antibodies with the history of thrombosis, thrombocytopenia, and fetal loss (antiphospholipid syndrome).

The laboratory determination of anti-β2GPI antibodies offers the following advantages. 1) Anti-β2GPI testing provides additional relevant information, along with classic antiphospholipid assays for assessing the antiphospholipid syndrome. 2) Because phospholipids are not used in this assay system, it is expected to improve the interlot and interlaboratory variability that has been observed with classic antiphospholipid assays. 3) The testing will significantly reduce or eliminate the number of false-positive results with antiphospholipid antibodies not associated with thrombosis that are occasionally produced by infections (i.e., syphilis). 4) The use of purified human β2GPI favors the detection of those "autoimmune" antibodies (increased sensitivity) that require this protein for optimal binding and are considered pathogenic for thrombosis.

References

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